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Methods for use of the single-electrode voltage clamp

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In order to obtain reliable data with the single-electrode voltage clamp, proper controls and test procedures must be done. The switching frequency and duty cycle are set according to the requirements of the cells and the experiment. Electrodes are used only if they can follow the switching frequency chosen and can pass required amounts of current without rectification. In each cell, the clamping ability of the electrode is tested with a calibrator signal.

CONTENTS

INTRODUCTION

Voltage clamping is a very important technique in neurophysiology. It provides a direct method of studying both intrinsic membrane currents and transmitter-induced or drug-induced currents. Until recently, many cells were unavailable for voltage clamping because their small size prevented impalement with two electrodes. A single-electrode circuit (SEC) was developed by W. A. Wilson (1), which provided a means of voltage clamping with only one electrode. Dunn and Wilson (2) demonstrated that the SEC could be used to voltage clamp cat spinal motoneurons in vivo. With the advent of a commercially available SEC (Dagan 8100, Axoclamp 2), a number of laboratories have applied this technique to their preparation (3-8).

Because the SEC works on a different principle than do more conventional recording techniques, the control and test procedures also differ. With the SEC, electrode characteristics are very important to the proper functioning of the system, and they must be carefully monitored. This report describes the procedures necessary to ensure meaningful clamp records. It is not intended to be a theoretical analysis of clamp limitations. Also, it is not meant to be an operating manual for the single-electrode clamp, but rather a guide for the experimenter once the hardware has been mastered.

SPACE CLAMP CONSIDERATIONS

Because of the complex microanatomy of most neurons in the central nervous system, electrophysiological recordings reflect only electrically local events. Electrical signals gradually decay with distance from the site of initiation. Whether attempting to voltage clamp or just to record membrane potential, the synaptic responses originating on a distant dendritic spine will be attenuated at the cell body. When injecting current to depolarize or hyperpolarize membrane potential, the cell will not be isopotential; the shift in membrane voltage will decline with distance from the electrode. These problems are present with or without voltage clamping and with both the one-electrode and two-electrode clamp systems.

With voltage clamping, it is important to keep in mind that remote sites will not see the full extent of a voltage command, and they cannot be adequately clamped to prevent spontaneous fluctuations in potential. Events initiating in the remote regions will not be under good voltage control, and will not be accurately

represented by recordings in the soma. For further discussion of the consequences of inadequate space clamp, the reader is referred to Johnston and Brown (9).

SWITCHING CIRCUIT

The single-electrode circuit requires one electrode to do the work of two. This is accomplished through a switching circuit that alternates between a current-injecting mode and a voltage-recording mode. The percentage of time spent injecting current is called the duty cycle. On the Dagan 8100, a choice of two duty cycles is provided: 50% and 25%. Near the end of the current-injecting period, the "sample and hold" circuit samples the current. This value is provided to the continuous current output until the next sample is taken. Similarly, the voltage is sampled at the end of each voltage-recording period.

The rate at which the clamp alternates between the two modes is called the switching frequency. The clamp relies on the time constant of the cell (membrane resistance times membrane capacitance) to smooth out the injected square waves of current and to provide essentially a steady DC current across the cell membrane. If the switching frequency is much slower than the time constant of the cell membrane, the injected current will have a substantial AC component at the switching frequency. To minimize the AC component, the switching cycle should be as fast as possible.

The switching frequency also limits the time resolution of the recordings. Electrophysiological events with higher frequency components will not be faithfully reproduced. For example, if the frequency is set at 5 kHz, voltage is sampled only once every 0.2 ms. An event faster than 2 ms probably will not be accurately represented.

If the headstage output and the unsampled current are directly monitored, one can observe the consequences of switching. In Figure 1, the top trace is the unsampled current. For 50% of the time, no current is injected through the electrode; the rest of the time, hyperpolarizing current flows. The lower trace is the headstage output. When no current is passed, the electrode records zero voltage; when current is injected, a voltage drop occurs across the electrode resistance. In Figure 1B, the duty cycle was changed to 25%. To inject the same amount of net current, the electrode must carry twice as much current for half the time.

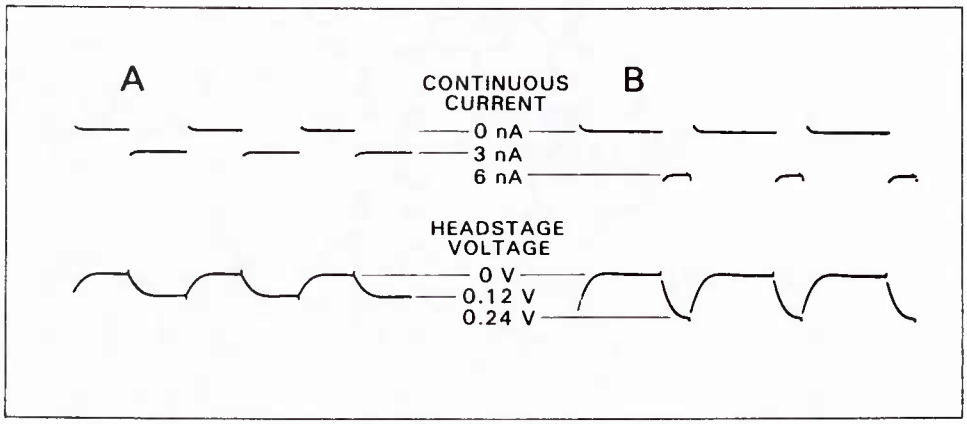

Figure 1. Effect of duty cycle on continuous current and headstage output of single-electrode voltage clamp. Top traces are oscilloscope tracings (sweep speed .1 ms/div) of continuous current. Bottom traces are unsampled headstage output. (A) Using 50% duty cycle, 3nA are injected through electrode to pass average of 1.5 nA of current. Voltage deflection across electrode resistance results in square wave on headstage output. Ratio of voltage change to current injected provides measure of electrode resistance, in this case 40 Mohms. (B) Using a 25% duty cycle, 6 nA must be passed to provide an average current of 1.5 nA. Similarly, deflection of headstage voltage is increased due to electrode resistance. A 25% duty cycle provides a longer voltage recording period, allowing more time for voltage to reach a steady-state level before sample is taken. Switching frequency in this and all other figures is 3 kHz.

Notice that the voltage does not return to baseline level instantaneously. A capacitance is associated with the electrode that requires charging and discharging. The voltage must return to baseline levels before the beginning of the next current injection, or incorrect voltage levels will be sampled. A 25% duty cycle allows 75% of the switching period for voltage levels to return.

Under some experimental conditions, the time constant associated with the electrode and wiring is large enough to prevent complete decay of the electrode voltage. Efforts should be made to minimize capacitance of the recording system. Some factors can be controlled, such as the capacitance added by high levels of saline solution or long wires. Often electrode capacitance can be reduced by shielding down the shank with silver paint. Connecting the driven shield of the clamp to the Faraday cage around the electrophysiological setup may help reduce stray capacitance.

The capacitance feedback control of the amplifier can compensate for some capacitance of the system. However, it has limitations. Microelectrodes are frequently associated with several time constants, some of which can be slow and unresponsive to capacitance compensation. In addition, increasing capacitance feedback can accentuate high frequency noise. It is preferable to minimize capacitance rather than compensate for it.

The most common cause of incomplete return of voltage during the duty cycle is the large time constant associated with high-resistance electrodes. These are often necessary for the impalement of small cells. In this situation, voltage clamping may be possible only if the switching frequency is low, to allow more time for the voltage to return to its steady-state level. But it is necessary to remember that switching frequency must be much faster than the time constant of the cell and that a reduced frequency limits the time response of the clamp, allowing only slower events to be accurately controlled.

ELECTRODE CONSIDERATIONS

As indicated above, the quality of the electrodes is crucial to adequate clamp operation. Ideally, electrodes should be very low in resistance and capacitance to provide the best clamp. However, less than ideal electrodes can be used, provided they pass a stringent series of tests.

Once the duty cycle and switching frequency are set, the capacitance feedback should be adjusted to compensate for system capacitance. This procedure should cause the headstage output waveform to become more square. With an electrode in the grounded extracellular solution, passing a small amount of steady current (from the internal source) produces a square wave that can be observed on the oscilloscope screen using a sweep speed of 0.2 ms/div and triggering off the continuous current trace or the switching frequency monitor. As shown in Figure 2, the headstage waveform may not look like a square wave. If the capacitance feedback is set too low, the voltage may never reach a plateau during the voltage-recording mode (trace 3) as discussed above. If it is set too high, oscillations can occur (trace 4). In either case, the voltage reading at the end of the voltage-recording period is erroneous. Again, it is important to set the capacitance compensation to a level that allows the voltage (which developed across the electrode while passing current) to decay completely. There will be a range of acceptable capacitance feedback settings. Within this range, changing the capacitance feedback will not affect sampled voltage. Some electrodes cannot be "squared up" and cannot be used.

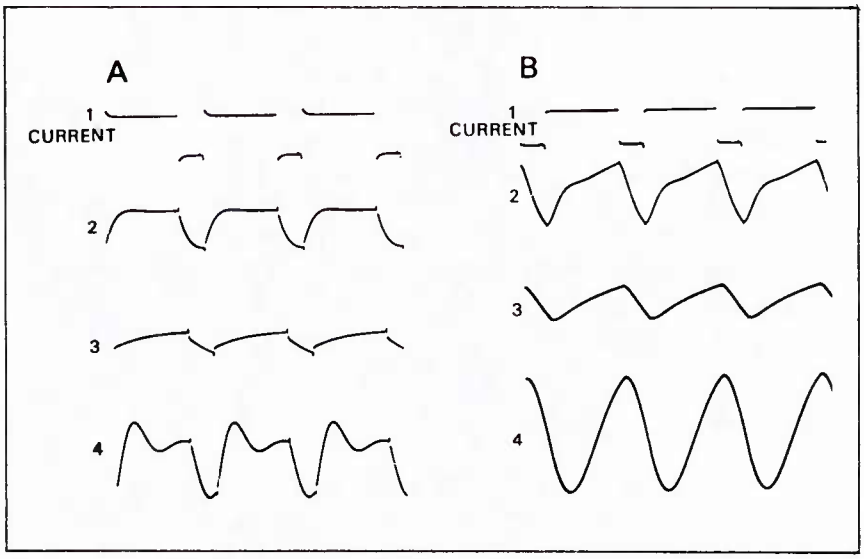

Figure 2. Appearance of headstage output provides important information on electrode. (A) Effect of adjusting capacitance feedback. All traces in A are from same electrode. Trace 1, continuous current record. Trace 2, headstage voltage with capacitance feedback properly adjusted. Trace 3, headstage voltage with undercompensation of capacitance. Trace 4, headstage voltage with overcompensation of capacitance. Only in trace 2 does voltage reach a steady-state level during voltage-recording period. (B) Examples of poor electrodes that cannot be squared up with capacitance feedback. Trace 1, continuous current record. Traces 2-4, three different electrodes that could not be used with single-electrode circuit because they could not follow switching frequency.

An electrode used with the single-electrode circuit must be tested for its ability to pass current. With the electrode in the extracellular solution, the offset voltage should be adjusted to ground level. Under these conditions, injecting current should not alter the sampled voltage. An electrode must be tested with at least as much current (both hyperpolarizing and depolarizing) for at least as long in duration as will be required during the course of an experiment. Ideally, the sampled voltage will not change at all. Practically, though, usually there is at least a small deviation from recorded ground, especially with larger currents. This reflects rectification of the electrode. Tolerance limits should be set, based on the experimental requirements. If the electrode rectifies more than can be tolerated, it should be replaced. Another useful approach is to accept some rectification in the electrode but to subtract it from the measured response of the cell. To do this, one should measure the potential developed across the electrode for various currents both before and after the intracellular recording. In this way, the current-voltage (I-V) relationship of the electrode is determined. This I-V curve should be subtracted from the I-V curve of the cell.

Usually an electrode rectifies in such a way that negative current produces a small hyperpolarization. On occasion, however, inverse rectification can be observed; positive current produces a hyperpolarization. This effect is more frequent with higher resistance electrodes. It should be noted that rectification can occur in the ground return of the system. Rectification problems can often be corrected by replacing the electrode or agar bridge used to reference the bathing solution to ground potential. Ensuring well-chlorided wires in the electrode and the ground return also can improve current-passing capabilities.

VOLTAGE CLAMPING

After a cell is impaled, the electrode may change some of its characteristics, often increasing in both resistance and capacitance. Usually after impaling a cell, capacitance feedback again requires adjustment to allow the headstage potential to reach a steady state during the voltage-recording period. The headstage output and continuous current traces reveal much about how the electrode is performing and changing. It is quite useful to keep an eye on these traces throughout an experiment. The process of adjustment is continuous. For example, capacitance can change with varying fluid levels, and may require readjustment to allow continued accurate monitoring of membrane voltage. During the course of an experiment, if the electrode changes in such a way that it can no longer be "squared up," then the electrode can no longer be used with confidence, and it should be discarded. In some situations, "buzzing" the electrode by transiently increasing capacitance feedback will clear an electrode. However, by doing this, one risks losing the impalement or injuring the cell.

Once an electrode that has met all requirements is in a cell, voltage clamping can be attempted. To avoid an immediate oscillation of the clamp, the gain should be turned down before the voltage clamp is switched on. As the gain is slowly increased, the injected current begins to fluctuate and the headstage output begins to look somewhat unstable (Figure 3). Adjustment of the phase control sometimes allows clamp gain to be further increased. The higher the gain of the clamp, the more accurately the membrane voltage will follow a voltage command. To test the adequacy of the clamp, a calibration pulse can be applied between the cell and ground. Ideally, a calibration pulse of 20 mV or greater should be completely clamped; that is, the membrane potential (sampled) of the cell will not change at all, although the membrane current will (Figure 4). In practice, some voltage deflection is likely to occur, especially with negative calibration pulses (equivalent to depolarizing steps eliciting large outward currents).

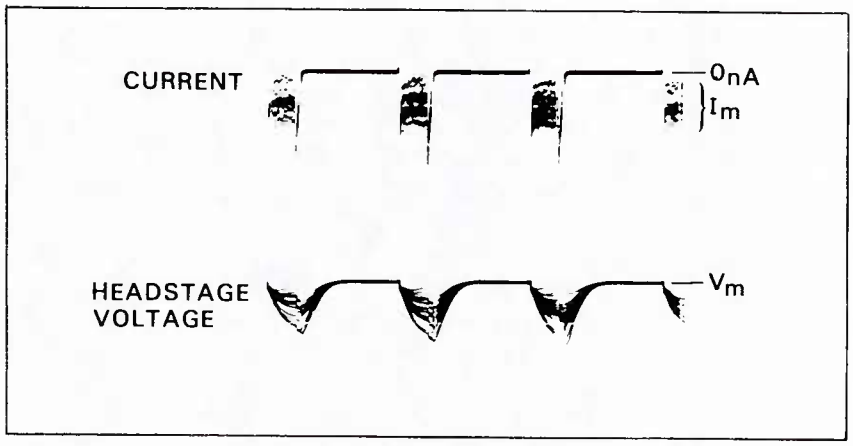

Figure 3. Continuous current and headstage voltage while voltage clamping. Variability of signal during current-passing phase increases with increasing clamp gain. Current injected is sampled and provides membrane current trace. At end of voltage-recording period, headstage voltage will be membrane potential.

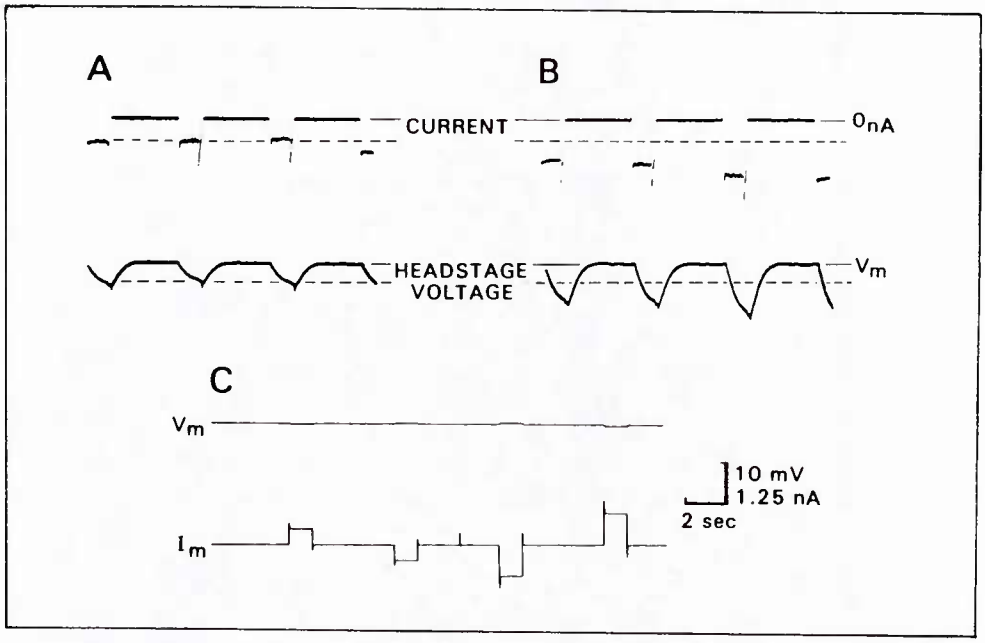

Figure 4. Test of clamping ability of electrode. While in a cell, a calibrator pulse is applied between bathing solution and ground. Since command voltage is unchanged, clamp will attempt to maintain same membrane potential. Sampled voltage trace should not change. Representative continuous current and headstage voltage traces before (A) and during (B) 20-mV calibrator pulse. Current is injected to clamp out applied voltage. Voltage drop across electrode is increased because of increased current, but sampled voltage is minimally altered. (C) Sampled membrane potential (top) and membrane current (bottom) during calibrator pulses of -10 mV, +10 MV, -20 mV, and +20 mV. No deflection of membrane potential is apparent with 10-mV calibrator pulses, but a small deflection is seen with 20-mV pulses.

Deviation under these conditions indicates that with an equivalent voltage command, voltage control would not be perfect. As before, tolerance levels need to be set, based on the experimental requirements. If the electrode does not meet the standards and if further increasing the clamp gain is not possible, then the electrode should be replaced. During the course of an experiment, the electrode characteristics and clamping ability might change, necessitating a decrease in clamp gain to maintain the cell under voltage clamp without oscillation.

Again, it should be noted that changing the gain on the voltage clamp can alter quantitative data. At lower clamp gains, membrane potential will not follow exactly the voltage command. A voltage command to a particular membrane potential may not agree with the voltage actually realized. For example, a hyperpolarizing command of 40 mV may actually elicit a hyperpolarization of only 37 mV. For this reason, it is important to monitor the actual membrane potential and not just the command potential. In addition, a voltage command that evokes large transient currents may not result in constant membrane potential throughout the step. If a transient outward current is evoked, for instance, the potential realized early in a depolarizing step may be less positive than later in the step.

At the termination of an experiment, when the electrode is removed from the cell, the electrode characteristics should again be checked. The DC offset and rectification properties should be noted. If changes were large, the data obtained might not be reliable.

DISCUSSION

Using the single-electrode clamp is no more difficult than using a bridge balance. However, the controls that are to be done to ensure that the equipment accurately depicts the events of the cell are much different. With the SEC, good electrodes are crucial. They must be capable of passing adequate current without much rectification. The electrode capacitance must be sufficiently low to allow complete discharge during the voltage-recording period at the chosen switching frequency. If an electrode cannot meet these criteria, it must be discarded. Once a cell is impaled, the voltage-clamping ability of the electrode can be tested by providing a calibrator signal between the cell and ground. With experience, these tasks become just as routine as balancing a bridge circuit.

Despite excellent electrodes and apparently good clamping, there are still pitfalls to the use of a voltage clamp. One must keep in mind that a clamp uses a point source of current to control a cell that in many cases has a complex microanatomy. Space clamping therefore becomes a problem because the cell is not isopotential. Electrical events initiated remote from the recording site will not be well clamped. Active currents such as those underlying the sodium and calcium action potentials can invade the clamped area of a cell and appear in the current records. If an event of interest occurs in an electrically remote part of the cell, a true representation of the currents is not obtained. The implications of inadequate space clamping in hippocampal neurons are well discussed by Johnston and Brown (9).

Another factor that is important to keep in mind is the limitation on the time resolution of the single-electrode clamp. Since it works through a switching circuit, it is much slower than a two-electrode clamp system in following a voltage command. While a two-electrode system continually compares actual membrane voltage to the command voltage, the SEC can compare the two values only when a sample of membrane is obtained, that is, once each cycle. Therefore, very fast currents are difficult to follow, if not impossible. A faster switching frequency can improve the clamp speed, but it requires electrodes with a very low time constant. Poor electrodes can sometimes be used at a slower switching frequency, but more high frequency response is lost.

The method presented here is not necessarily the only way to use the single-electrode voltage clamp. Other laboratories (3, 4, 7) have developed their own techniques for ensuring adequate clamping. It is often useful to validate these techniques in a system that allows impalement with a second electrode. Since that is not always possible, a well tried method is convenient, such as that presented here. Any method of testing for adequate clamping must take into account that the electrode (a) has adequate frequency response to follow the switching frequency, and (b) is capable of passing sufficient current without rectification. Failure to satisfy these two criteria can result in misleading data.

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